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Applicant(s): Martin Frederick Pera — **Examiner:** J. Woitach

Serial No.: 09/885,679 — **Art Unit:** 1632

Filed: June 20, 2001 — **Docket:** 14727

For: IMPROVED METHODS OF CULTURING EMBRYONIC STEM CELLS AND CONTROLLED DIFFERENTIATION — **Dated:** December 4, 2003

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

CLAIM OF PRIORITY

Sir:

Applicant in the above-identified application hereby claims the right of priority in connection with Title 35 U.S.C. §119 and in support thereof, herewith submits a certified copy of Australian Patent Application PQ 8242, filed on June 20, 2000 and Australian Patent Application PR 1327, filed on November 8, 2000.

Respectfully submitted,

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CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner For Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on December 4, 2003.

Dated: December 4, 2003

Frank S. DiGiglio

AUSTRALIA

Patent Office
Canberra

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 8242 for a patent by HADASIT MEDICAL RESEARCH SERVICES and DEVELOPMENT CO., NATIONAL UNIVERSITY OF SINGAPORE and MONASH UNIVERSITY filed on 20 June 2000.

I further certify that the name of the applicant has been amended to HADASIT MEDICAL RESEARCH SERVICES and DEVELOPMENT CO. LTD, NATIONAL UNIVERSITY OF SINGAPORE and MONASH UNIVERSITY pursuant to the provisions of Section 104 of the Patents Act 1990.

WITNESS my hand this
Twenty-ninth day of June 2001

J R Yabsley

JONNE YABSLEY
<u>TEAM LEADER EXAMINATION</u>
<u>SUPPORT AND SALES</u>

P/00/009
Regulation 3.2

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: IMPROVED METHODS OF CULTURING EMBRYONIC STEM CELLS

Applicant: HADASIT MEDICAL RESEARCH SERVICES AND DEVELOPMENT CO. Ltd, NATIONAL UNIVERSITY OF SINGAPORE, AND MONASH UNIVERSITY

AUSTRALIAN PATENT OFFICE
SEC 104

The invention is described in the following statement:

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IMPROVED METHODS OF CULTURING EMBRYONIC STEM CELLS

The present invention relates to a method of culturing embryonic stem cells particularly to improve stem cell maintenance and persistence in culture. The method also provides a culture of embryonic stem cells prepared by the method and differentiated cells derived from the embryonic cells.

INTRODUCTION

The production of human embryonic stem cells which can be either maintained in an undifferentiated state *or* directed to undergo differentiation into extraembryonic or somatic lineages *in vitro* allows for the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug screening and drug discovery *in vitro*.

In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a haematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any cell.

Much attention recently has been devoted to the potential applications of stem cells in biology and medicine. The properties of pluripotentiality and immortality are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. ES cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. However, it must be noted that almost all of the wide ranging potential applications of ES cell technology in human medicine-basic embryological research, functional genomics, growth factor and drug discovery, toxicology, and cell transplantation-are based on the assumption that it will be possible to grow ES cells on a large scale, to introduce genetic modifications into them, and to direct their differentiation. Present systems fall short of these goals.

Present systems for the growth of human embryonic stem cells include the use of Dulbecco's modified Eagle's medium as a basal media with the addition of amino acids and beta mercaptoethanol, serum supplementation, and embryonic mesenchymal feeder cell support. Growth under these conditions is not sufficient for many applications including scaleup of cultures and cloning of single cells, the latter being necessary for selection of transformants following genetic manipulation. Moreover, under present growth conditions, stem cells often follow a default pathway of differentiation into an epithelial cell type that grows either as flat squamous cells attached to the surface of the dish, or in cysts. It is likely that this form of differentiation represents extraembryonic endodermal differentiation and it is postulated that this cell type resembles the primary yolk sac cells of the primate embryo. As noted previously, conditions in which this form of differentiation predominates are unfavourable for differentiation of ES cells into desired somatic cell types.

Accordingly, it is object of the present invention to overcome or alleviate some of the problems of the prior art.

SUMMARY OF THE INVENTION

In a first aspect of the present invention there is provided a preparation of undifferentiated embryonic stem cells sustainable for a prolonged period in an undifferentiated state. Preferably the cells are capable of somatic differentiation *in vitro*.

Embryonic stem cells have a natural capacity to differentiate into cells similar to those found in extraembryonic endodermal lineages of the early embryo. Accordingly, if the cells are not treated to prevent this default differentiation pathway, somatic lineages cannot be effectively attained or maintained *in vitro* for further studies or manipulation. It is desired to maintain the cells in an undifferentiated state giving greater capacity for manipulation of differentiation particularly into somatic lineages.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of a cell derived insulin or insulin analogue induced factor.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of insulin or an insulin analogue.

In yet another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of a fibroblast and insulin or an insulin analogue.

Accordingly, this aspect of the invention provides a method for culturing embryonic stem cells so as to enhance stem cell survival and growth during routine culture and to enhance the extent and variety of somatic cells obtained under differentiation conditions.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells capable of somatic differentiation *in vitro*, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of a cell derived insulin or insulin analogue induced factor.

Preferably the cells deriving the factor are fibroblasts or embryonic stem cells.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells capable of somatic differentiation *in vitro*, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of fibroblast and insulin or an insulin analogue.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells capable of somatic differentiation *in vitro*, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of insulin or an insulin analogue.

In another aspect of the present invention, there is provided a cell derived insulin or insulin analogue induced factor capable of maintaining embryonic stem cells in an undifferentiated state but favouring somatic differentiation. The cells deriving the factor may be fibroblasts or cultures containing embryonic stem cells.

In another aspect there is provided a differentiated committed progenitor cell line capable of differentiation into somatic cells.

In another aspect, there is provided a somatic cell capable of differentiation *in vitro* from an undifferentiated embryonic stem cell. There is also provided a committed somatic cell capable of giving rise to mature somatic cells. The cells may differentiate into embryonic mesoderm and embryonic endoderm lineages, including, but not limited to cartilage, muscle, bone, hepatocyte, pancreatic islet cells and respiratory endothelium.

FIGURES

Figure 1A shows a graph of the number of cells/colony obtained when ES cells are cultivated under control conditions or in the presence of insulin for 7 days. The percentage of cells reactive with stem cell markers TRA-1-60 and GCTM-2 is shown in Figure 1B.

Figure 2 shows agarose gel electrophoresis analysis of RT-PCR for Oct-4, GCNF, cripto, GDF-3, genesis, and β actin on control ES cell cultures (A) ES cell cultures maintained in insulin for five passages.

Figure 3 shows phase contrast morphology of differentiating ES cell cultures grown in control medium (A) or in the presence of insulin (B-D). Control cultures consist mainly of squamous epithelial cells which form cysts, while insulin tested cultures retain a range of cell types.

Figure 4 shows agarose gel electrophoresis of RT-PCR analysis for markers of endodermal lineages obtained from stem cells or spontaneously differentiating ES cells. Markers include alphafetoprotein, HNF3 alpha, transferrin, vitronectin. Actin is shown to indicate RNA quantity.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect of the present invention there is provided a preparation of undifferentiated embryonic stem cells sustainable for a prolonged period in an undifferentiated state. Preferably the cells are capable of somatic differentiation *in vitro*.

Embryonic stem cells have a natural capacity to differentiate into cells resembling those of extraembryonic endodermal lineages in the early human embryo. Accordingly, if the cells are not treated to prevent this defaulted differentiation pathway, somatic lineages cannot be effectively attained or maintained *in vitro* for further studies or manipulation. It is desired to maintain the cells in an undifferentiated state giving greater capacity for manipulation of differentiation particularly into somatic lineages.

The purified preparation of the present invention is capable of prolonged cultivation and are substantially maintained under conditions which do not induce cell death or of differentiation. However, it is preferable that the cells are capable of differentiation particularly toward somatic lineages such as when a differentiating signal, is introduced.

Preferably, the embryonic cells are capable of maintaining an undifferentiated state when cultured on a fibroblast feeder layer generally under non-differentiating conditions. Desirably the fibroblast feeder layer does not induce cell death or extraembryonic differentiation.

The cultured cells maintained in the undifferentiated state may have the potential to differentiate *in vitro* when subjected to differentiating conditions. However when a differentiation signal is given it is best that the cells have the capacity to differentiate *in vitro* into a wide array of somatic lineages.

The promotion of stem cells capable of being maintained in an undifferentiated state *in vitro* on one hand, and which are capable of differentiation *in vitro* into somatic lineages on the other hand, allows for the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug screening and drug discovery *in vitro.*

Once the cells are maintained in the undifferentiated state, they may be differentiated to mature functional cells. The embryonic stem cells are derived from the embryo and are pluripotent and have the capability of developing into any organ or tissue type such as blood cells, neuron cells or muscle cells.

The type of undifferentiated embryonic stem cell and methods of obtaining such cells is described in PCT/AU99/00990. The entire contents of that application are incorporated herein.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of a cell derived insulin or insulin analogue induced factor.

Preferably the cells deriving the factor are fibroblasts or cultures containing embryonic stem cells.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of insulin or an insulin analogue.

In yet another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of a fibroblast feeder layer and insulin or an insulin analogue.

Accordingly, this aspect of the invention provides a method for cultivation of embryonic stem cells so as to enhance stem cell survival and growth during routine culture and to enhance the extent and variety of somatic cells obtained under differentiation conditions.

In a preferred aspect of the invention, there is provided a method of culturing undifferentiated embryonic stem cells, said method including:

obtaining a source of undifferentiated embryonic stem cells;

culturing the embryonic stem cells on a fibroblast feeder cell layer; and

subjecting the cultured cells to an effective amount of insulin or an insulin analogue.

The cells may be cultured on fibroblast cells and then subjected to insulin or an insulin analogue possibly after the cells have been initially cultured for a time to establish the ES cell culture.

The Applicants have found a means to achieve this by culturing the embryonic stem cells generally on a fibroblast feeder cell layer in the presence of insulin or an insulin analogue. The analogue may be IGF-I or IGF-2. Preferably the insulin analogue is IGF-2. The insulin or an analogue may induce the fibroblasts or the stem cells to produce a factor(s) capable of supporting stem cell growth and which favours somatic differentiation.

Insulin or analogues thereof including IGF-1 or IGF-2 have been used to support the growth and survival of a wide range of cultured mammalian cells. However, the positive effects of insulin or analogues thereof including IGF-1 or IGF-2 on growth and survival of embryonic stem cells cultured in the presence of serum and in the presence or absence of a feeder cell support have not been reported.

The action of insulin or an insulin analogue such as IGF-2 may be directly on the embryonic stem cells, or indirectly through the action of insulin or an analogue on a feeder cell layer (if present) or by a combination of these two mechanisms. Insulin or an analogue may function in combination with factors produced by the fibroblasts, or by stem cells themselves, such as Cripto or GDF-3.

For instance, where the embryonic stem cells are grown in the absence of a feeder cell layer, the cell derived insulin or insulin analogue induced factor may be harvested from a separate culture of cells such as fibroblasts exposed to insulin or insulin analogue. The conditioned medium derived from this source containing the insulin or insulin analogue induced factor may then be introduced to the ES culture at a concentration suitable to maintain the cells in an undifferentiated state but capable of differentiation into a somatic lineage. Alternatively, the ES cells may be grown on a fibroblast feeder cell layer and the insulin or insulin analogue may act on the ES cells themselves, in conjunction with factors produced by the fibroblasts or by the stem cells themselves. The growth factors GDF-3 and cripto are produced by human ES cells. Addition of these factors along with insulin or insulin analogue may further enhance cell

growth. Other factors may be produced by insulin or insulin analogue treated stem cells which will enhance stem cell growth or survival.

Insulin or an insulin analogue such as IGF-2 may be added at a concentration of approximately 10 ng/ml to 10μg/ml to the fibroblast culture from which the factor is liberated and preferably harvested. It may also be added to an ES cell culture maintained on a fibroblast feeder cell layer which liberates the factor *in situ* for immediate benefit, or to act on the ES cells directly. Ideally, the concentration is 10μg/ml.

The insulin or an analogue may be added at the time of cultivation either directly to the ES cells on the fibroblast feeder cell layer or to a fibroblast culture from which the conditioned medium is harvested or during a process of obtaining the ES cells. The insulin or an analogue may be added alone or in combination with other factors such as GDF-3 or Cripto.

In another aspect of the present invention, there is provided a cell derived insulin or insulin analogue induced factor capable of maintaining ES cells in an undifferentiated state but capable of differentiation into a somatic lineage. Preferably the factor is induced by subjecting fibroblasts or embryonic stem cells to insulin or analogues thereof. The factor is generally found in the culture medium (supernatant) of fibroblasts or embryonic stem cells and may be isolated therefrom.

The cell derived insulin or insulin analogue induced factor from fibroblasts may be identified by the following:

1) Treating a feeder cell layer with insulin or an analogue , harvesting conditioned medium from treated or control cells, testing the biological effects of these media on ES cells, and identifying the active factors by biochemical means known to the skilled addressee such as by chromatography by testing each factor; or

2) Differential analysis of gene expression in control cells such as fibroblasts and fibroblasts treated with insulin or analogue to identify these factors using for example representation of display analysis followed by standard techniques of molecular cloning and recombinant protein expression, or related techniques known to the skilled addressee.

Likewise, ES derived insulin or insulin analogue induced factors might be identified by the following:

1) Treating the ES cells with insulin or an analogue, harvesting conditioned medium from treated or control cells, testing the biological effects of these media on ES cells, and identifying the active factors by biochemical means known to the skilled addressee such as by chromatography by testing each factor; or

2) Differential analysis of gene expression in control ES cells and ES cells treated with insulin or an analogue to identify these factors using for example representational display analysis followed by standard techniques of molecular cloning and recombinant protein expression, or related techniques known to the skilled addressee.

The ES cells may be grown in the presence or absence of a feeder cell layer. Where a feeder cell layer is used, it is preferably a fibroblast feeder cell layer of the type described in PCT/AU99/00990. The entire contents of that application are incorporated herein.

The effects of insulin or an analogue on the fibroblast derived insulin induced factor may be evident within 5 to 7 days of subcultivation of an ES colony in media containing insulin.

In another aspect of this invention there is provided populations of stem cells or committed progenitor cells identified by particular patterns of surface antigen expression, including but not limited to those which are positive for both GCTM-2 and TRA 1-60, those which are positive for one or other of these antigens, and those which lack either antigen on their surface

The effect of insulin or analogue or the insulin or insulin analogue induced factor is evidenced morphologically by a more tightly packed and uniform appearance compared to colonies grown in the absence of insulin or analogues or the factor. Many more cells of uniform stem cell morphology are present in treated colonies, and immunostaining methods using stem cell specific markers to count the number of cells expressing such markers or estimating their proportion by flow cytometry using such stem cell specific markers may be used to confirm this. Without being limited by theory, it is considered that the chief effect of insulin or analogue treatment or the effect of the factor over the short term is to increase the number of cells in each colony

without a marked effect on the proportion of stem cells present, consistent with an effect on stem cell multiplication or survival.

Applicants have observed that there is heterogeneity even in cultures consisting of cells with the appearance of stem cells. For example, the proportion of cells reactive with the markers GCTM-2 is consistently lower than that of cells reacting with TRA 1-60. This may reflect stem cells at different levels of maturation within the population. Insulin or analogue treatment consistently produces a modest decrease in the proportion of TRA 1-60 positive cells but does not change the proportion of GCTM-2 positive cells. This may be indicative of a change in the rate of stem cell maturation. Isolation of the various subpopulations of cells (eg TRA1-60 + GCTM-2 +, GCTM-2 + TRA1-60-, TRA 1-60 + GCTM-2 -, or TRA 1-60 – GCTM-2 -)in control and insulin or analogue treated cultures may help identify novel cellular intermediates with desired properties, such as enhanced colony forming ability or somatic differentiation capacity.

It has now been found that stem cells grown in the presence of insulin or analogue or the insulin or analogue induced factor persist much longer than stem cells grown in the absence of insulin or analogue or of the factor. They may still be present up to 3 weeks or more whilst control colonies (grown in the absence of insulin or the factor) will consist of differentiated cells.

Undifferentiated stem cells may be propagated and subcultured for multiple passages in the presence of insulin or analogue or the factor. Successful long term maintenance of stem cells in the presence of insulin or analogue or the factor may be proven by the continued presence in the cultures of diploid cells bearing stem cell markers and expressing stem cell specific genes such as Oct-4. Furthermore, in cultures passaged through 20 to 30 population doublings, stem cells may be demonstrated by such cells forming teratomas in SCID mice which contain derivatives of all three embryonic germ layers.

Hence insulin or an analogue or the cell derived insulin or insulin analogue induced factor is useful as described in the present invention to increase the number and persistence of stem cells during routine passage.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells capable of somatic differentiation *in vitro*, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of a cell derived insulin or insulin analogue induced factor.

Preferably the cells deriving the factor are fibroblasts or embryonic stem cells.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells capable of somatic differentiation *in vitro*, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of fibroblast and insulin or an insulin analogue.

In another aspect of the present invention there is provided a method of culturing undifferentiated embryonic stem cells capable of somatic differentiation *in vitro*, said method including:

obtaining a source of undifferentiated embryonic stem cells; and

culturing the embryonic stem cells in the presence of insulin or an insulin analogue.

In addition to affecting the number and persistence of stem cells during routine passage, the addition of insulin or analogue or the factor can also affect the outcome of differentiation.

In a preferred aspect of the invention, there is provided a method of culturing undifferentiated embryonic stem cells capable of somatic differentiation *in vitro*, said method including:

obtaining a source of undifferentiated embryonic stem cells;

culturing the embryonic stem cells on a fibroblast feeder cell layer; and

subjecting the cultured cells to an effective amount of insulin or an insulin analogue.

The cells may be cultured on fibroblast cells and then subjected to insulin or an insulin analogue possibly after the cells have been initially cultured for a time to establish the ES cell culture.

Somatic differentiation is favoured under conditions that limit stem cell renewal but support cell survival and limit extraembryonic differentiation. Applicants have found that addition of insulin to fibroblast feeder cells limits extraembryonic differentiation into the extraembryonic endodermal cell type. The decreased proportion of cells undergoing extraembronic differentiation may be demonstrated by the lower proportion of cells bearing characteristic markers of this lineage. The phenotypic identification of the extra embryonic cell may be demonstrated by the presence of specific markers of the extraembryonic endodermal lineage, using immunocytochemistry or RT-PCR, such markers including transcription factors such as HNF3 alpha and beta, cytokeratins including cytokeratin 19, the cell adhesion molecule vitronectin and basement membrane molecules such as Type IV collagen and laminin. Stem cells persist longer in cultures treated with insulin or an analogue thereof or the fibroblast derived insulin or insulin analogue induced factor and as such cultures may be maintained without renewal of a feeder layer, somatic differentiation is strongly favoured, with neuroectoderm precursors (staining for N-CAM and nestin and expressing Pax-6) appearing first, in larger numbers than in control dishes. Thereafter, many additional cell types may be seen in insulin or insulin-induced factor treated cultures that are not usually detected in control cultures due to high proportions of extraembryonic cells in the latter. These novel cell types may be characterised by distinctive morphology and patterns of gene expression.

In another preferred embodiment, the undifferentiated cells cultured according to the invention differentiate under differentiating conditions *in vitro* to form somatic lineages.

In another aspect there is provided a differentiated committed progenitor cell line capable of differentiation into somatic cells.

In another aspect, there is provided a somatic cell capable of differentiation *in vitro* from an undifferentiated embryonic stem cell. There is also provided a committed somatic cell capable of giving rise to mature somatic cells. The cells may differentiate into embryonic mesoderm and embryonic endoderm lineages, including, but not limited to cartilage, muscle, bone, hepatocyte, pancreatic islet cells and respiratory endothelium.

These cells may be obtained by somatic differentiation of human ES cells, identified by markers. These cells may be isolated in pure form from differentiating ES cells, *in vitro*, and propagated *in vitro.* They may be induced to undergo differentiation to mature somatic cell lineages.

In the presence of a differentiation signal, undifferentiated ES cells in the right conditions will differentiate into derivatives of the embryonic germ layers (endoderm, mesoderm and ectoderm), and/or extraembryonic tissues such as neuron tissue. This differentiation process can be controlled.

Conditions for obtaining differentiated cultures of somatic cells from embryonic stem cells are described in PCT/AU99/00990, the contents of which are incorporated herein.

Once the cells have been induced to differentiate, the various cell types, identified by means described above, may be separated and selectively cultivated. The progenitor cells may differentiate into any cells including embryonic mesoderm and embryonic endoderm lineages, including, but not limited to cartilage, muscle, bone, hepatocyte, pancreatic islet cells and respiratory endothelium.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any was as a restriction on the generality of the invention described above.

EXAMPLES

Example 1: Action of insulin on short term maintenance of human embryonic stem cells.

HES-1 or HES-2 cells described in PCT/AU99/00990 were subcultured in the presence or absence of insulin and maintained without subculture for periods of 1-4 weeks. Colonies of treated or control cells were harvested using dispase. Some colonies were used to determine the presence of transcripts for stem cell specific genes (Oct-4, Cripto, Genesis, GDF-3, GCNF) in the cultures by RT-PCR. Other colonies were dissociated to single cells and stained for the presence of stem cell specific markers such as the GCTM-2 antigen. The total number of cells per colony was determined and the percentage of stem cells

present was assessed by flow cytometry. The effect of insulin on stem cell survival was assessed by assay of apoptosis on colonies. (See Figures 1 and 2).

Example 2: Action of insulin on long-term maintenance of human embryonic stem cells.

HES 1 and HES-2 were cultivated for 10 passages in the presence of insulin. At this point, the cell phenotype was determined using immunochemistry and RT-PCR for stem cell markers, the karyotype was assessed by G-banding, and the ability to differentiate into various cell types was assessed by transplantation of cells into said mice and by immunostaining of cells grown to high density for various markers of specific differentiated cell types including neuronal and muscle cells.

Example 3: Effect of insulin on somatic differentiation of human ES cells.

HES1 and HES2 were subcultured in the presence of insulin and maintained for 3-6 weeks without further transfer. The extent of extraembryonic differentiation was assessed by morphological evaluation of the presence of squamous cells forming cystic vesicles. The extent of somatic differentiation was assessed by morphological assessment and by immunochemical staining for known markers of somatic cell lineages including neuronal markers. (See Figures 3 and 4).

Example 4: Basis of the action of insulin.

To assess whether the actions of insulin were direct or mediated through the feeder cell layer, the following studies were carried out. The presence of receptors for insulin or IGFs on the stem cells was determined using RT-PCR or immunochemistry. The actions of insulin on stem cells grown in the absence of a feeder layer was determined using the methods described in 1 above. The effect of insulin treatment of the feeder layer was determined by treating a feeder cell layer with insulin, collecting the supernatant, removing the low molecular weight proteins including insulin using ultrafiltration, and testing the high molecular weight fractions for support of stem cell growth as described above (1); alternatively, an insulin treated feeder cell layer was extracted to

yield an extracellular matrix preparation and stem cells were cultivated on this preparation and assessed for growth as described above (1). Identification of specific genes expressed in insulin treated feeder cell layers was carried out using standard molecular biology methods for comparison of gene expression in related cell types, such as representational display analysis.

Example 5: Isolation of novel subsets of stem cells with desired properties. Cells from control or insulin treated cultures were harvested, stained with antibodies GCTM-2 and TRA 1-60, and populations of cells bearing either marker only, both markers, or neither marker, were isolated by immunomagnetic beads or flow cytometry. These cells were cultured, and their colony forming ability and differentiation capacity was assessed. (See Figure 1).

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

DATED: 20 June, 2000
PHILLIPS ORMONDE & FITZPATRICK
Attorneys for:
MONASH INSTITUTE OF REPRODUCTION & DEVELOPMENT

David B Fitzpatrick

Figure 1A

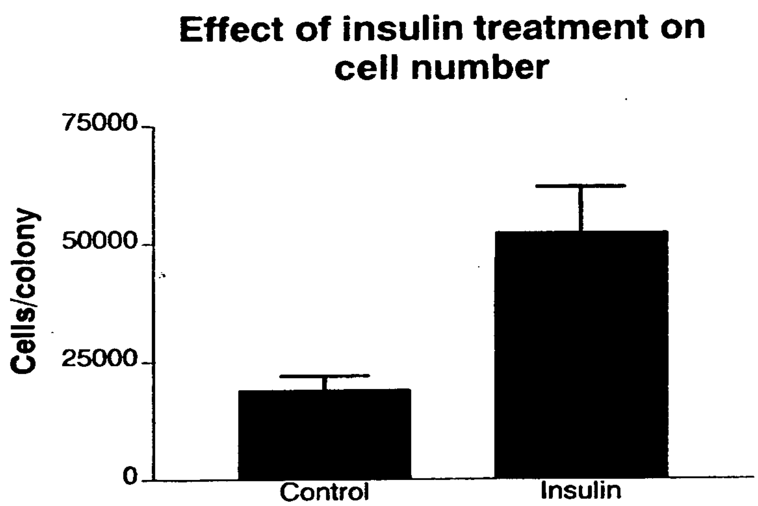

Effect of insulin treatment on cell number
Cells/colony
75000
50000
25000
0
Control
Insulin

Figure 1B

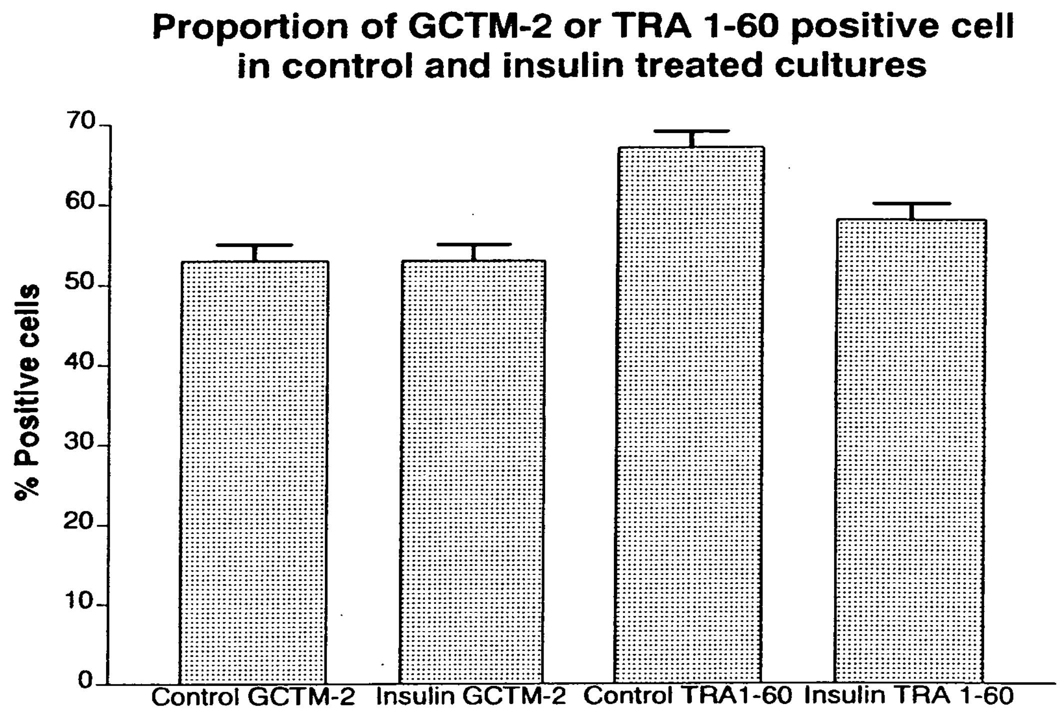

Proportion of GCTM-2 or TRA 1-60 positive cell in control and insulin treated cultures
% Positive cells
70
60
50
40
30
20
10
0
Control GCTM-2
Insulin GCTM-2
Control TRA1-60
Insulin TRA 1-60

Figure 2

Gene Expression in insulin treated HES

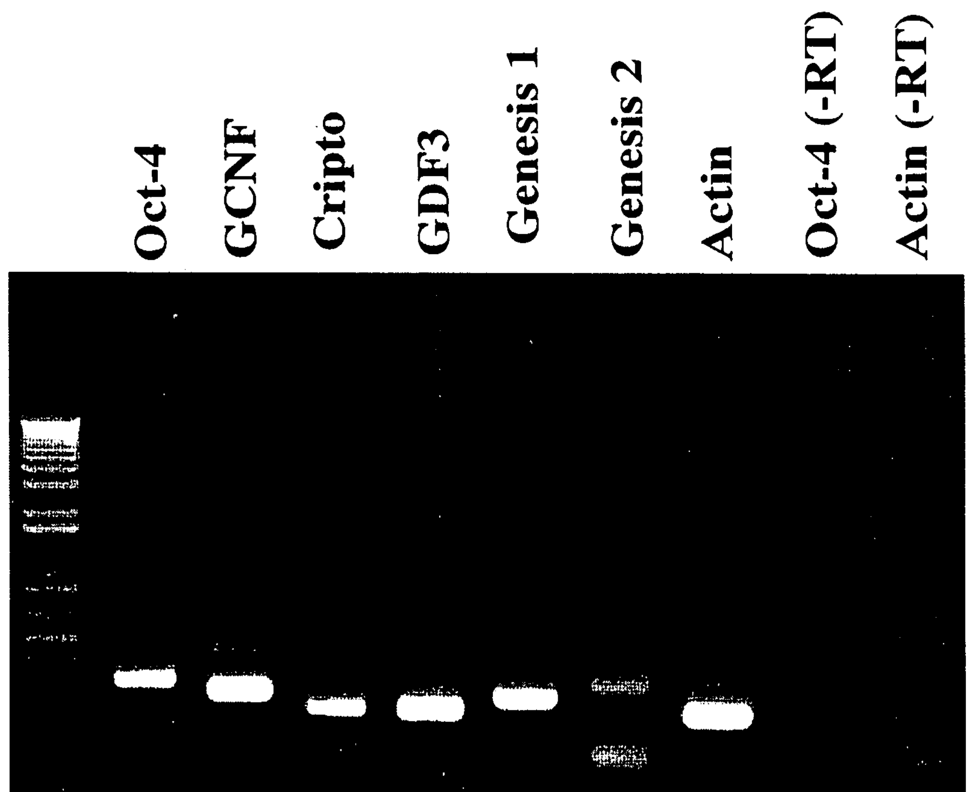


Figure 3A

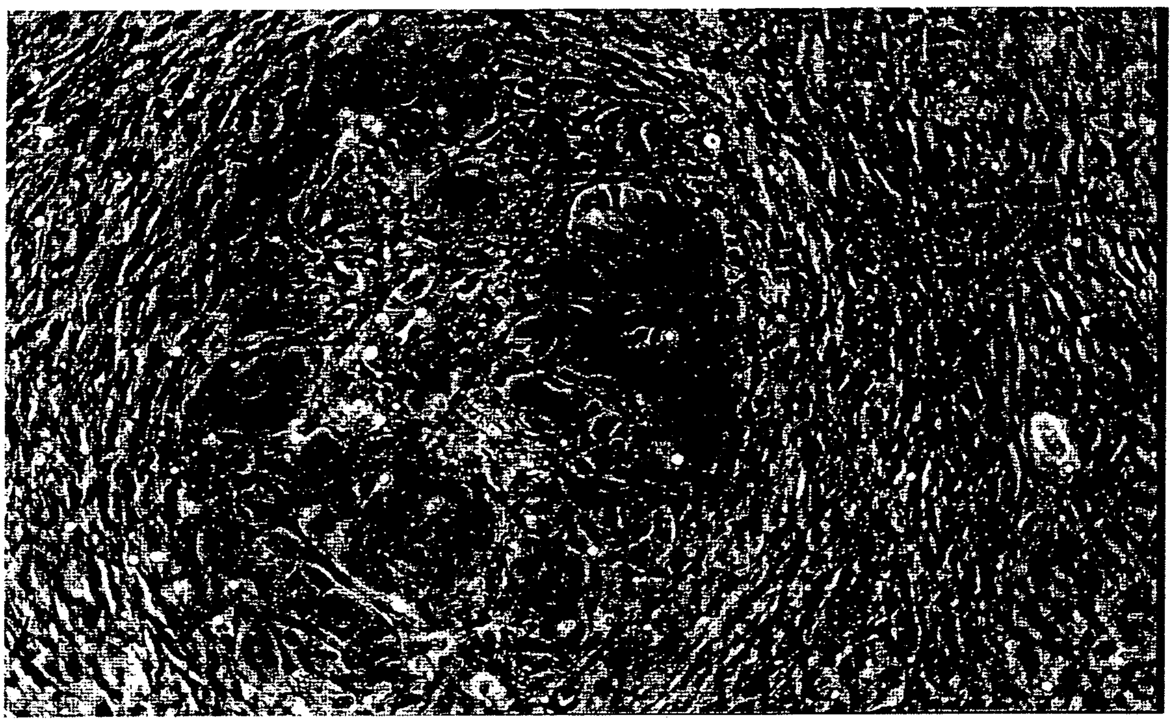


Figure 3B

Figure 3C

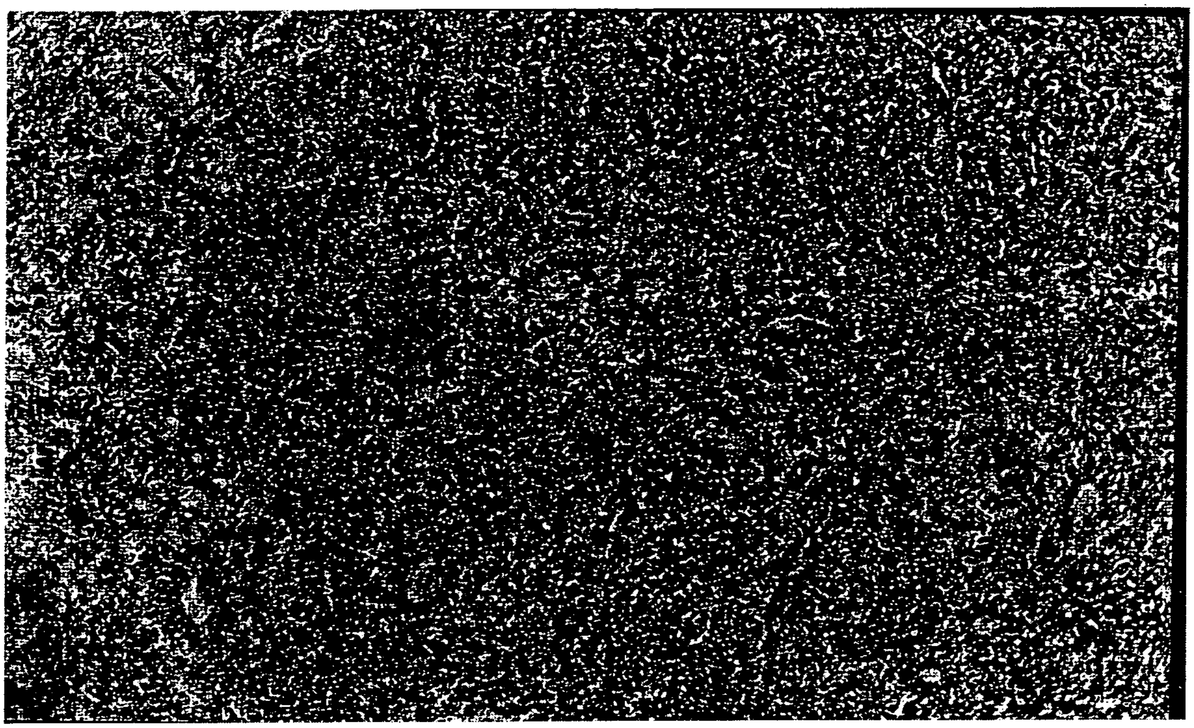


Figure 3D

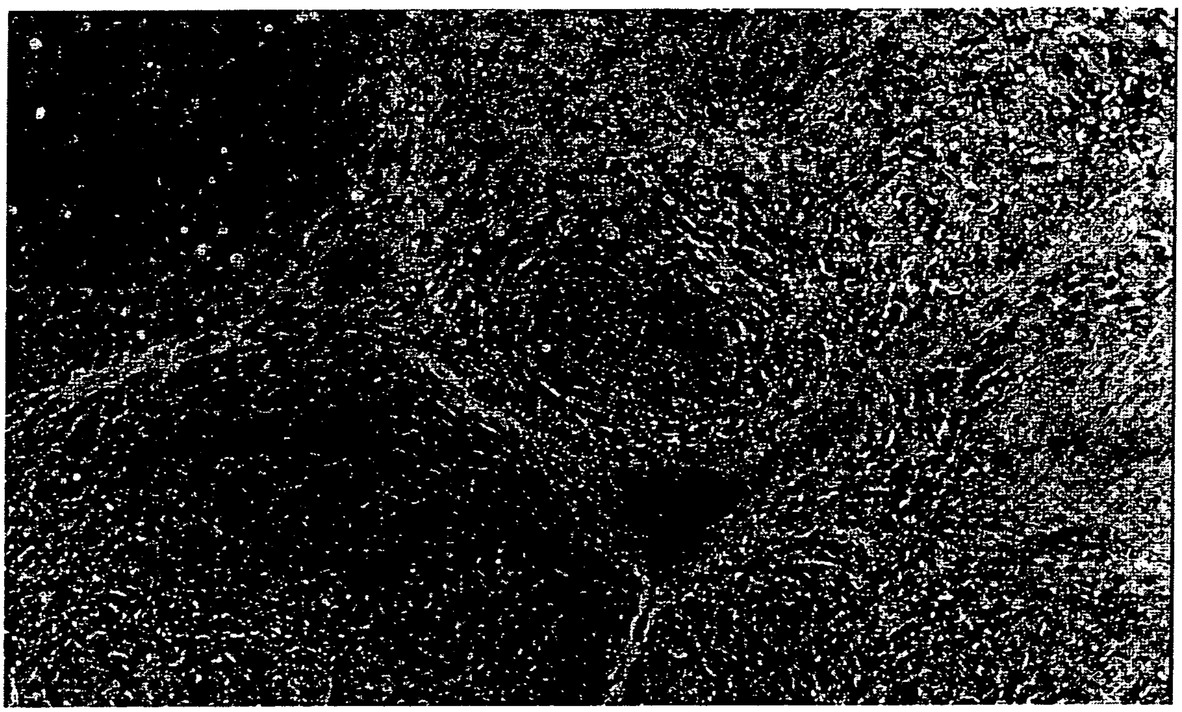
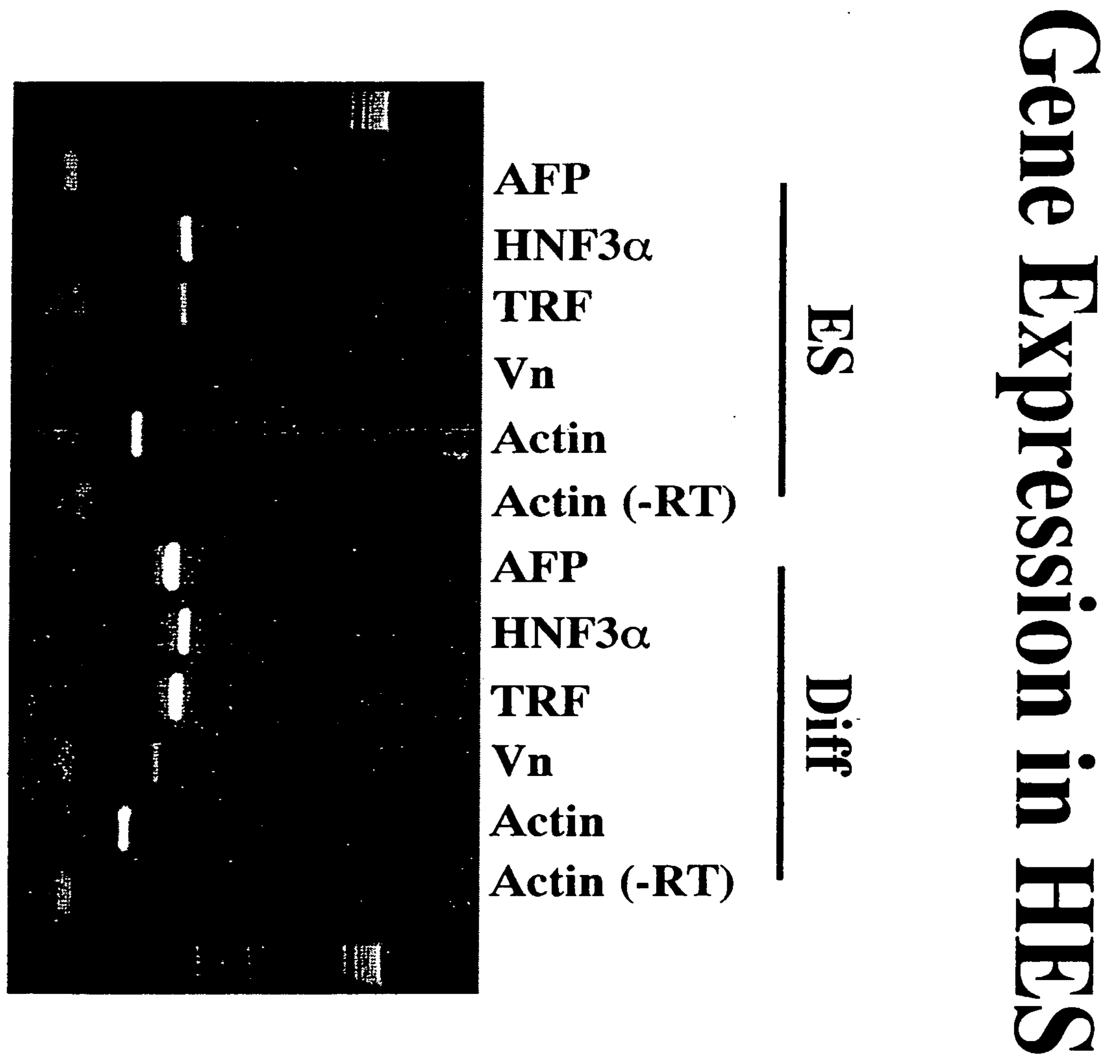


Figure 4


Gene Expression in HES
ES
Diff
AFP
HNF3α
TRF
Vn
Actin
Actin (-RT)
AFP
HNF3α
TRF
Vn
Actin
Actin (-RT)